

Elucidation of anthracyclinone biosynthesis by stepwise cloning of genes for anthracyclines from three different *Streptomyces* spp.

Jaana Kantola,¹ Tero Kunnari,² Anne Hautala,² Juha Hakala,² Kristiina Ylihonko^{1,2} and Pekka Mäntsälä¹

Author for correspondence: Kristiina Ylihonko. Tel: +358 2 3336879. Fax: +358 2 3336860.
e-mail: kristiina.ylihonko@finabo.abo.fi

¹ Department of Biochemistry, University of Turku, Vatselantie 2, FIN-20014 Turku, Finland

² Galilaeus Oy, PO BOX 113, FIN-20781 Kaarina, Finland

The anthracycline skeleton is biosynthesized by aromatic (type II) polyketide synthases. Furthermore, three post-polyketide steps are needed to form the basic aglycone of anthracyclines. Auramycinone was produced in *Streptomyces lividans* by introducing nine structural genes from three different anthracycline-producing *Streptomyces* species. The genes used to construct the auramycinone biosynthesis cluster were derived from nogalamycin-, daunomycin- and aclacinomycin-producing *Streptomyces* strains. The biosynthetic stages were divided into polyketide and post-polyketide steps on the assumption that the first stable intermediate would be nogalonic acid, named analogously to aklanonic acid, the precursor of several anthracyclines. Single genes were cloned in the expression construct in the order determined by the proposed biosynthetic pathway. This facilitated investigation of the products formed in the heterologous host after addition of each separate gene to the construct. The results thus elucidate the biosynthesis steps, products and the genes responsible for the reactions needed to build up an anthracyclinone.

Keywords: anthracyclines, auramycinone, biosynthesis, heterologous expression, *Streptomyces*

INTRODUCTION

Anthracyclines are aromatic polyketide antibiotics produced by *Streptomyces* species and consist of an aglycone skeleton and sugar moieties (Fig. 1). Diversity arises from structural differences in the aglycone and from a wide array of attached sugar residues. The significant antitumour activity of anthracyclines makes them commercially interesting and indeed anthracyclines have been successfully used in cancer chemotherapy for decades. Anthracyclines are divided into several subgroups, of which the daunomycins are the best known, since all of the clinically used anthracyclines except aclacinomycin A belong to the daunomycin group.

Auramycins are related in structure to aclacinomycin (Fujiwara *et al.*, 1982) and are produced by a mutant

strain OBB-111, derived from *Streptomyces galilaeus* ATCC 31533, which accumulates aclacinomycins. The only difference between auramycins and aclacinomycins is the alkyl group at position 9 (Fig. 1). Probably, biosynthesis proceeds in the same manner as in aclacinomycins though the starter unit in biosynthesis is different.

The organization of the polyketide synthase (PKS) gene cluster of *S. galilaeus* has been reported recently (Fujii & Ebizuka, 1997) and the molecular genetics of daunomycin biosynthesis has been intensively studied by Hutchinson, Strohl and their coworkers (e.g. Madduri & Hutchinson, 1995; Rajgarhia & Strohl, 1997). Related work on other aromatic polyketides has added to our knowledge of biosynthetic reactions in the anthracycline pathway (e.g. Hutchinson & Fujii, 1995; McDaniel *et al.*, 1995; Hopwood, 1997; Zawada & Khosla, 1997).

We report here a step-by-step introduction of single genes for anthracyclinone biosynthesis into a plasmid construct in the order predicated by the proposed biosynthetic pathway (Ylihonko *et al.*, 1996b). The compounds accumulated in a heterologous host were

Abbreviations: AAME, aklanonic acid methyl ester; minPKS, minimal PKS; PKS, polyketide synthase.

The GenBank accession number for *acmA* reported in this paper is AF043550.

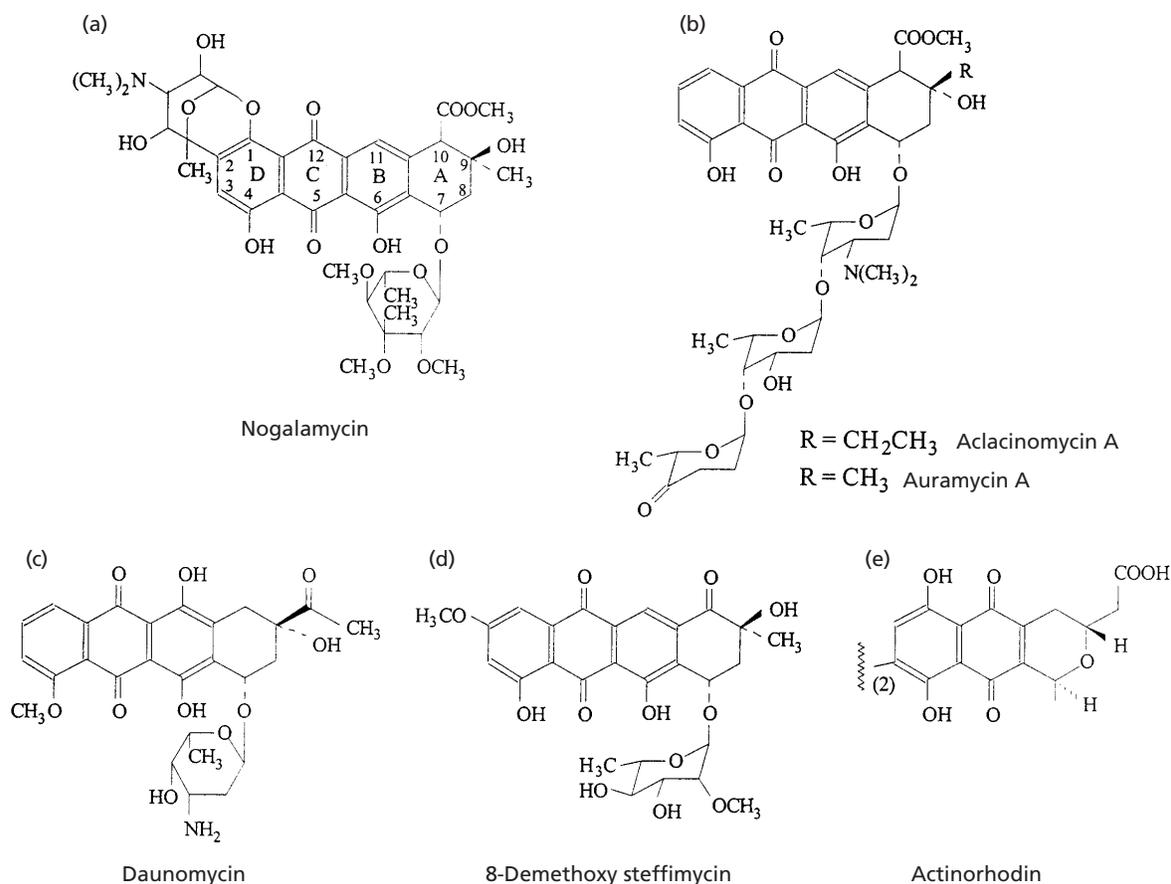


Fig. 1. The structures of (a) nogalamycin, (b) aclacinomycin A and auramycin A, (c) daunomycin, (d) 8-demethoxy steffimycin and (e) actinorhodin.

analysed after each step. The choice of *Streptomyces lividans* as the host for expression cloning was based on evidence that it does not itself accumulate anthracycline metabolites in the culture conditions used for fermentation.

METHODS

Bacterial strains and plasmids. Manipulations of *Streptomyces* DNA were carried out in *Escherichia coli* XL2-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacI^qZ ΔM15 Tn10(Tet^r) Amy Cam^r*]) (Stratagene). *Streptomyces* strains used were *Streptomyces nogalater* ATCC 27451 (Bhuyan & Dietz, 1965), *Streptomyces steffisburgensis* ATCC 27466 (Dietz, 1967), *S. galilaeus* ATCC 31615 (Oki *et al.*, 1975), the blocked *S. galilaeus* mutants H036, H039 and H061 (Ylihonko *et al.*, 1994), *Streptomyces peucetius* ATCC 27952 (Arcamone *et al.*, 1969) and *S. lividans* TK24 (Hopwood *et al.*, 1985). The plasmids used are listed in Table 1.

Culture conditions and cloning procedures. DNA propagated in *E. coli* was ligated into pIJ486 derivatives and introduced by protoplast transformation into *S. lividans* TK24. DNA isolation and manipulation were carried out by standard procedures (Sambrook *et al.*, 1989; Hopwood *et al.*, 1985). *Streptomyces* were transformed by standard methods (Hopwood *et al.*, 1985), with minor modifications (Ylihonko *et al.*, 1996a).

Strains were cultivated in 60 ml E1 medium for 5–6 d (28 °C, 330 r.p.m.) to produce polyketides. Products were isolated by extracting with chloroform/methanol (3:1, v/v) for TLC and HPLC analysis.

Expression constructs. Plasmids were made in pSY21 carrying the nogalamycin minimal PKS (minPKS) genes or in pSY15 expressing the genes for the nogalamycin chromophore (Ylihonko *et al.*, 1996b). MinPKS and an activator from the *S. nogalater* gene cluster were cloned in the *Streptomyces* vector pIJ486 as a 5.5 kb *SacI*–*Bgl*III fragment to give pSY21 (Fig. 2). A 12 kb *Bgl*III fragment from the *sno* cluster was cloned in pIJ486 to give pSY15 (Fig. 2). According to Table 1, suitable restriction sites were used for cloning. The restriction sites were made blunt-ended by treatment with Klenow polymerase to make pSY21a, pSY15a and pSY15b. Furthermore, the inserts introduced into the vector to generate pSY21b, pSY21c and pSY21d were cloned through the *E. coli* vector pSL1180 to add convenient restriction sites. The gene for aklanonic acid methyl ester cyclase was subcloned from pAcmA, derived from the *S. galilaeus* gene cluster and the gene encoding aklaviketone reductase was subcloned from pDx2 carrying a 2.2 kb DNA fragment of the daunomycin gene cluster cloned from *S. peucetius*. All plasmids were introduced into *S. lividans* strain TK24. Constructs are listed in Table 1.

Isolation and purification of products. Strains were grown in a 10 l fermenter (E1 medium, 28 °C, 500 r.p.m.) for 6 d.

Table 1. Plasmids used in this study

sno, *acm* and *dau* indicate that the gene was cloned from *S. nogalater*, *S. galilaeus* and *S. peucetius*, respectively. ARO, aromatase; KR, polyketide ketoreductase; OXY, 12-mono-oxygenase; MET, nogalonic acid 14-methyltransferase.

Plasmid	Relevant characteristics	Reference or source
pSL1180	<i>E. coli</i> cloning vector	Brosius (1989)
pUC19	<i>E. coli</i> cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pIJ486	<i>Streptomyces</i> cloning vector	Ward <i>et al.</i> (1986)
pSY21 (10–12)*	Expression vector for <i>sno</i> minimal PKS	Ylihonko <i>et al.</i> (1996b)
pSY15 (1–12)*	Causes production of the nogalamycin chromophore	Ylihonko <i>et al.</i> (1996a)
pAcmA	<i>acmA</i> (AAME cyclase) in pUC19	This work
pDx2	<i>dauE</i> (aklaviketone reductase) in pUC19	This work
pSY21a (3–6)*	<i>snoD</i> (KR) added to pSY21	This work
pSY21b (2–5)*	<i>snoD</i> (KR) and <i>snoE</i> (ARO) added to pSY21	This work
pSY21c (7–9)*	<i>snoB</i> (OXY) added to pSY21b	This work
pSY21d (4–8)*	<i>snoB</i> (OXY) and <i>snoC</i> (MET) added to pSY21b	This work
pSY15a	<i>acmA</i> (AAME cyclase)† added to pSY15	This work
pSY15b	<i>dauE</i> (aklaviketone reductase)† added to pSY15a	This work

* Restriction sites used for cloning are indicated by the numbers in parentheses. The restriction sites correspond to Fig. 2a.

† Restriction sites used for cloning are indicated by asterisks in Fig. 2b, c.

Compounds were extracted separately from the mycelium and supernatant with dichloromethane/methanol (3:1, v/v) at pH 3.0. Solvents were removed under vacuum and the viscous residue was chromatographed through a polyamide 11 column using water/methanol from 1:9 to 0:10 (v/v) as eluent. Individual fractions were further purified using a preparative reversed-phase (C-18) column with acetonitrile/1% acetic acid in water (1:1, v/v) as mobile phase. Evaporation of the solvent under vacuum gave pure products.

Instrumental analysis. ^1H and ^{13}C NMR spectra using CDCl_3 or DMSO-d_6 as solvent were recorded on a JEOL JNM-GX400 spectrometer. NMR analysis included NON (1D proton spectra), BMC (1D carbon spectra), NOE (nuclear Overhauser effect), DEPT (distortionless enhancement by polarization transfer) and HMBC (heteronuclear multiple-bond connectivities) techniques. Spectra were internally referenced to tetramethylsilane. MS was performed on a Varian VG707E spectrometer. Metabolites were detected by HPLC (LaChrom, Merck Hitachi, pump L-7100, detector L-7400 and integrator D-7500) using a LiChroCART RP-18 column.

RESULTS

Cloning of anthracycline biosynthesis clusters

To clone single genes from different anthracycline pathways, gene clusters from *S. nogalater* (*sno*), *S. galilaeus* (*acm*) and *S. peucetius* (*dau*) (Fig. 2) were used. These strains produce nogalamycin, aclacinomycins and daunomycin, respectively (see Fig. 1 for structures). Aklavinone, the aglycone moiety of aclacinomycins, is

also a precursor of daunomycin, whereas the aglycone moiety of nogalamycin differs from aklavinone in the nature of its side chain and its configuration at C-9 (see Fig. 1 for differences).

A *sno* cluster containing the nogalamycin biosynthetic region was discovered by sequential use of the *actI* and *acm* probes (Ylihonko *et al.*, 1996a). The sequence is listed in GenBank under the accession number (Z48262)/AJ224512. It was used as the source of seven structural genes encoding activities needed for polyketide biosynthesis and of the gene responsible for esterification of the aklavinone analogue, nogalonic acid (Fig. 3). A fragment containing the gene required for each biosynthetic step was subcloned from the *sno* cluster using convenient restriction sites shown in Table 1 and Fig. 2(a).

The *actI* probe (Malpartida *et al.*, 1987) was used for hybridization to locate and clone the *acm* cluster from *S. galilaeus*. A 3 kb *Bam*HI fragment of the *acm* cluster ('*acm* probe' containing the gene *acmA*) encodes AAME cyclase responsible for closing the last ring of aklavinone (A-ring, Fig. 1a) in aclacinomycin biosynthesis. *acmA* was similar (83% similarity and 70% identity at the amino acid level) to *dauD* encoding AAME cyclase for daunomycin biosynthesis (Dickens *et al.*, 1995). The gene was inserted into pSY15 from the 3 kb *Bam*HI fragment using restriction sites *Eco*47III and *Bam*HI (Fig. 2b). The DNA sequence of *acmA* was deposited in GenBank under the accession number AF043550.

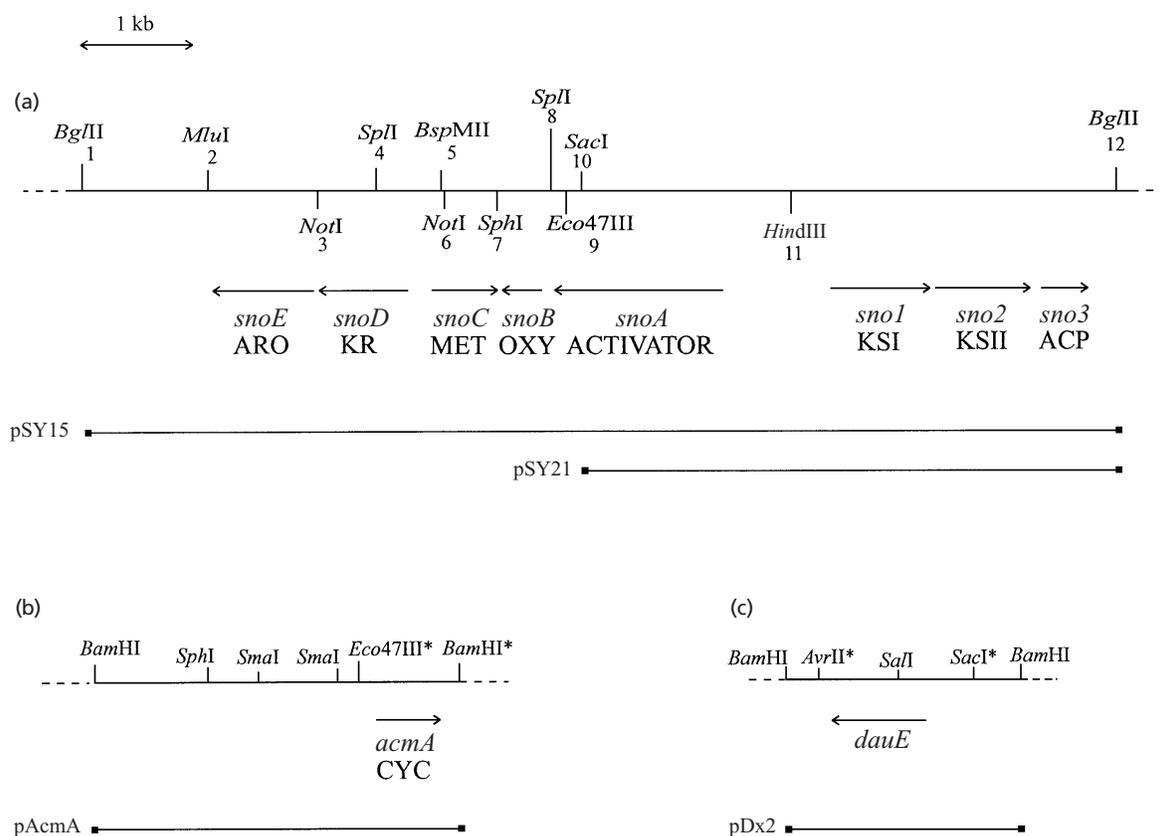


Fig. 2. The fragments of anthracycline biosynthesis clusters used in this study. (a) *S. nogalater* genomic DNA (*sno*) carrying part of the nogalamycin biosynthetic cluster. (b) *S. galilaeus* genomic DNA (*acm*) carrying part of aclacinomycin biosynthetic cluster. (c) *S. peuceetius* genomic DNA (*dau*) carrying part of daunomycin biosynthetic cluster. The ORFs are shown by arrows. DNA fragment inserts in pSY15, pSY21, pAcmA and pDx2 are indicated. The numbering of restriction sites used for cloning *sno* ORFs corresponds to that in Table 1. The restriction sites used for cloning *acmA* and *dauE* are marked with asterisks. Abbreviations of ORF functions: ARO, aromatase; KR, polyketide ketoreductase; MET, nogalonic acid 14-methyltransferase; OXY, 12-mono-oxygenase; KSI, ketosynthase I; KSIi, ketosynthase II; ACP, acyl carrier protein; CYC, aklanonic acid methyl ester cyclase. *dauE* is aklaviketone reductase.

A *dau* cluster covering about 60 kb contiguous DNA for daunomycin biosynthesis was located and cloned by hybridizing to the *acm* probe. A 2.2 kb DNA fragment (pDx2) of the *dau* cluster complemented H036, a *S. galilaeus* mutant accumulating aklaviketone. Sequencing and sequence analysis of pDx2 revealed one complete ORF named *dauE*, because the nucleotide sequence was 98% identical to the gene (*dauE*) previously characterized from *Streptomyces* sp. strain C5 (Dickens *et al.*, 1996) encoding aklaviketone reductase. A *SacI*–*AvrII* fragment of pDx2 (Fig. 2c) was used to introduce the C-7 ketoreductase gene into the final construct, pSY15b (Fig. 4).

Products generated by pSY21- and pSY15-based clones

Plasmids pSY21 and pSY15 were used as expression vectors into which the DNA fragments containing single specific genes were sequentially introduced. Plasmid pSY21 is the expression construct for nogalamycin minPKS and pSY15 contains the genes needed to form the nogalamycin chromophore (Ylihonko *et al.*, 1996b).

The route for preparation of constructs that finally resulted in pSY21d and pSY15b (Fig. 4) is shown in Table 1. The products from separate expression constructs were first studied in *S. lividans* TK24 and, whenever possible, the structures of the compounds accumulated were determined. Spectral data for structural elucidation of the compounds IVC, VA–VD, VI and VII are shown in Table 2.

Plasmids pSY21, pSY21a and pSY21b each caused the production of a confusing mixture of dozens of unstable compounds in *S. lividans*. No structural analysis of these compounds was carried out, since the relative amounts differed in separate fermentation batches. However, using the *S. galilaeus* blocked mutants and *S. steffisburgensis* as the hosts, the functionality of the various constructs was analysed.

Plasmid pSY21 was introduced into the *S. galilaeus* mutant H039, which produces aklavinone with attached rhodinose residues (Ylihonko *et al.*, 1994). When *S. nogalater* minPKS in pSY21 was expressed in H039, the hydrolysed products obtained were aklavinone and auramycinone, whereas aklavinone was obtained after

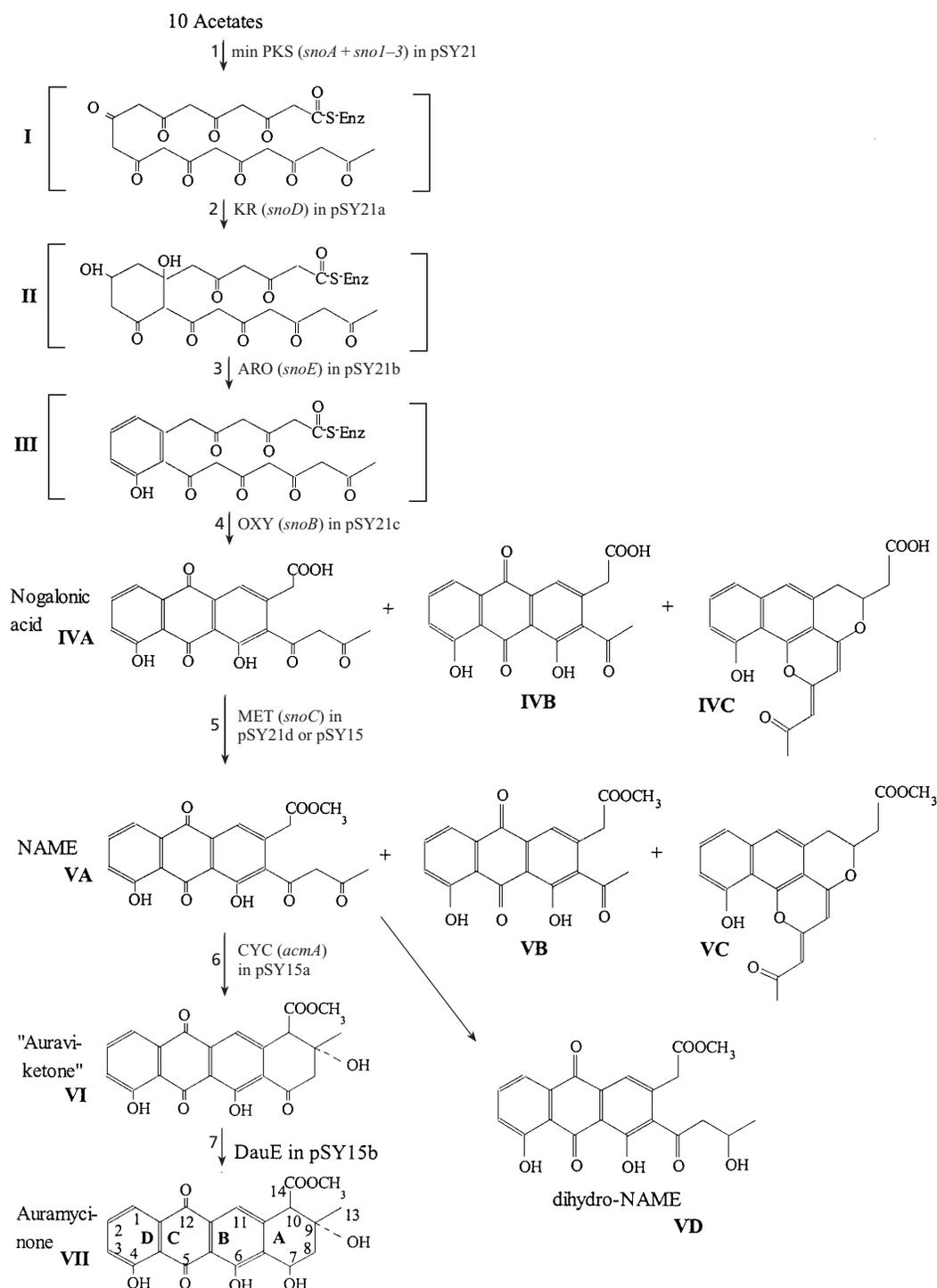


Fig. 3. Biosynthetic pathway for auramycinone. Reaction steps: 1. minPKS is responsible for forming a polyketide chain. 2. The ninth carbon from a carboxy terminus in an enzyme-bound complex is reduced by polyketide reductase (KR). 3. Aromatase (ARO) aromatizes the first ring. 4. The first stable intermediate is formed by the action of a 12-mono-oxygenase (OXY). 5. A methyl group is added to the carboxylic acid group by nugalonic acid 14-methyltransferase (MET) and the product is nugalonic acid methyl ester (NAME). 6. Cyclase (CYC) closes ring A. 7. Aklaviketone reductase (DauE) reduces a keto group at position 7, allowing the attachment of a sugar residue. Compounds I-III are postulated biosynthetic intermediates; other compounds have been identified in TK24/pSY clones. 'Auraviketone' is named by analogy with aklaviketone, the intermediate in aklavinone biosynthesis. NMR and MS data of the compounds identified are shown in Table 2.

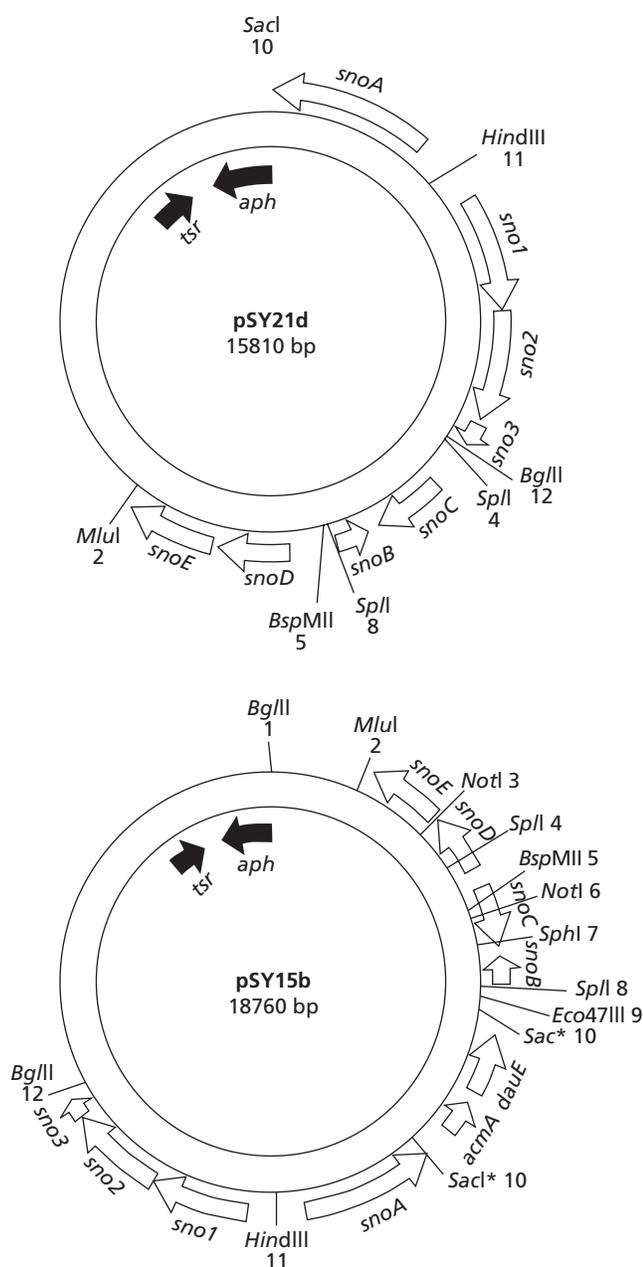


Fig. 4. Construction of pSY21d and pSY15b. ORFs are shown as arrows. The numbering of *sno* ORFs and the restriction sites correspond to those in Fig. 2a. *sno* indicates that the ORF was cloned from *S. nogalater*; *acm* from *S. galilaeus* and *dau* from *S. peucetius*. The gene for SnoA is complete in pSY15; it is incomplete but functional in all other constructs.

hydrolysis of H039 products. Aklavinone and auramycinone differ only in the ethyl or methyl substituent at C-9, respectively, due to use of a different starter unit in building the polyketide chain (Ylihonko *et al.*, 1996b).

Plasmid pSY21a was introduced into the polyketide-ketoreductase-deficient *S. galilaeus* mutant H061 for complementation. H061 was previously complemented to produce aklavinone glycosides by *snoD* encoding polyketide ketoreductase (KR) for nogalamycin bio-

synthesis (Ylihonko *et al.*, 1996b). When the hydrolysed products of H061/pSY21a were analysed, the aglycones obtained were aklavinone and auramycinone, as expected. H061 accumulates a 2-OH compound exhibiting a different folding pattern from anthracyclines (Kantola *et al.*, 1997).

S. steffisburgensis producing steffimycin (see Fig. 1 for the structure of 8-demethoxy steffimycin) was employed to analyse the effect of co-expression of aromatase with ketoreductase in pSY21b. We recently characterized the products of *S. steffisburgensis* (Kunnari *et al.*, 1997) and found that the main product of *S. steffisburgensis* (ATCC 27466) was 8-demethoxy steffimycin in the conditions used for fermentation. Steffimycinone differs from nogalamycin aglycone in position 2, the site for polyketide ketoreductase action (Figs 1 and 3) in nogalamycin biosynthesis. The absence of this step in the steffimycin pathway results in a methoxy group at C-2 in steffimycin. Expression of *snoD* and *snoE* in *S. steffisburgensis* caused the production of 2,8-demethoxy steffimycins (Kunnari *et al.*, 1997) and pSY21b generated the same products in this strain, confirming the functionality of the construct.

Plasmid pSY21c was the first construct that caused the production of stable compounds in TK24 and in the same proportion in separate fermentation batches. Structural analysis of these products revealed the aklanonic acid analogue nogalonic acid (IVA, Fig. 3) supporting the function of *snoB* as an oxygenase and suggesting that *snoB* is probably the last component in the PKS complex, as a stable compound was formed. Nevertheless, the major compound obtained (IVC) was a hybrid, perhaps caused by the concomitant action of cloned nogalamycin genes and the host's endogenous actinorhodin biosynthesis genes. Furthermore, a compound IVB of 18 carbons was also accumulated. IVB was suggested to be a biosynthetic product derived from nine acetates, presumably derived from the flexibility of minPKS in chain elongation of a growing polyketide chain. This was, however, a minor product. We have also found that some *S. galilaeus* mutants accumulating intermediates in aklavinone biosynthesis produce anthracycline metabolites derived from shorter polyketide chains than expected if the typical ten building blocks were used.

Plasmid pSY21d contains the same genes for biosynthesis of the aglycone as pSY15, and the product profiles of TK24/pSY21d and TK24/pSY15 were identical, as expected. The accumulation of nogalonic acid methyl ester (VA) demonstrated that *snoC* is responsible for methylation of a carboxy group at C-10. The structures of the compounds produced by TK24/pSY15 are shown in Fig. 3. All the compounds, including the intermediate IVA, the shunt product IVB and the hybrid product IVC, were converted to the methylated forms by the action of SnoC, resulting in VA, VB and VC, respectively. Furthermore, the reduced compound VD was accumulated in TK24/pSY21d as in TK24/pSY15. Because the product profiles caused by pSY15 and

Table 2. Spectral data of the compounds VII, VI, VA, VD, VB, IVC and VC shown in Fig. 3

(a) ^1H NMR (400 MHz) chemical shifts. The spectra were measured in CDCl_3 except VB, which was measured in $\text{DMSO}/\text{CDCl}_3$. All spectra were internally referenced to tetramethyl silane.

Site	VII	VI	VA	VD	VB	IVC	VC
1	7.69, 1H, dd, 7.5, 1.3	7.75, 1H, dd, 7.5, 1.2	7.85, 1H, dd, 7.5, 1.1	7.80, 1H, dd, 7.6, 1.2	7.85, 1H, dd, 7.4, 1.2	7.18, 1H, dd, 8.4, 1.0	7.19, 1H, dd, 7.8, 1.0
2	7.62, 1H, dd, 8.3, 7.5	7.65, 1H, dd, 8.4, 7.5	7.72, 1H, dd, 8.4, 7.5	7.70, 1H, dd, 8.4, 7.6	7.73, 1H, dd, 8.5, 7.4	7.38, 1H, dd, 8.4, 7.8	7.45, 1H, dd, 7.8, 7.3
3	7.23, 1H, dd, 8.3, 1.3	7.30, 1H, dd, 8.4, 1.2	7.33, 1H, dd, 8.4, 1.1	7.31, 1H, dd, 8.4, 1.2	7.34, 1H, dd, 8.5, 1.2	6.82, 1H, dd, 7.8, 1.0	7.03, 1H, dd, 7.3, 1.0
4-OH	11.78, 1H, s	12.59, 1H, s	11.94, 1H, s	11.85, 1H, s	11.95, 1H, s	11.16, 1H, s	11.43, 1H, s
6-OH	12.55, 1H, s	13.90, 1H, s	12.58, 1H, s	12.52, 1H, s	12.54, 1H, s	–	–
6a	–	–	–	–	–	5.90, 1H, s	5.75, 1H, s
7	5.33, 1H, dd, 5.1, 1.8	–	–	–	–	–	–
8A	2.58, 1H, dd, 14.9, 5.1	3.45, 1H, d, 17.6	5.94, 1H, s, (enol)	3.25, 1H, dd, 17.7, 2.7	2.96, 3H, s	5.44, 1H, s	5.38, 1H, s
8B	2.22, 1H, ddd, 14.9, 1.8, 1.5	2.83, 1H, dd, 17.6, 1.7	4.18, 2H, s, (keto)	3.11, 1H, dd, 17.7, 9.0	–	–	–
9	–	–	–	4.42, 1H, ddq, 9.0, 6.3, 2.7	–	–	–
9-OH	4.28, 1H, s	–	15.46, 1H, brs (enol)	–	–	–	–
10A	4.04, 1H, d, 1.4	4.18, 1H, d, 1.7	3.86, 2H, s	3.86, 1H, s	3.88, 2H, s	3.18, 1H, dd, 16.4, 3.1	3.20, 1H, dd, 16.0, 3.0
10B	–	–	–	–	–	3.00, 1H, dd, 16.4, 10.6	3.03, 1H, dd, 16.0, 10.2
10a	–	–	–	–	–	4.69, 1H, cm	4.73, 1H, cm
11A	7.55, 1H, s	7.71, 1H, s	7.77, 1H, s	7.68, 1H, s	7.71, 1H, s	2.83, 1H, dd, 16.1, 7.8	2.91, 1H, dd, 15.8, 7.2
11B	–	–	–	–	–	2.74, 1H, dd, 16.1, 7.8	2.75, 1H, dd, 15.8, 5.8
12	–	–	–	–	–	7.32, 1H, s	7.28, 1H, s
13	1.41, 3H, s	1.53, 3H, s	2.20, 3H, s, (enol) 2.32, 3H, s, (keto)	1.29, 3H, d, 6.3	–	2.03, 3H, s	2.15, 3H, s
15	3.70, 3H, s	3.76, 3H, s	3.72, 3H, s	3.73, 3H, s	3.72, 3H, s	–	3.77, 3H, s

(b) ^{13}C NMR (100 MHz) chemical shifts measured in CDCl_3 and internally referenced to tetramethyl silane.

Site	VII	VA	VD	IVC	VC
1	120.2	122.3	120.4	118.1	118.3
2	137.4	137.5	137.7	130.1	130.9
3	124.8	125.0	125.1	112.5	113.7
4	162.4	162.6	162.7	154.3	157.6
4a	115.5	115.6	115.6	111.5	112.6
5	192.3	184.4	192.6	149.9	155.5
5a	114.4	115.1	115.4	106.8	106.9
6	161.1	160.1	160.1	157.4	157.6
6a	133.2	132.2	135.2	96.8	97.6
7	62.4	192.5	205.7	161.7	162.2
8	37.0	103.8	52.6	96.6	97.1
9	69.9	191.3	64.3	192.8	194.3
10	57.8	39.3	38.8	39.2	39.6
10a	142.3	142.7	141.9	74.2	74.0
11	121.1	120.3	120.4	31.2	32.2
11a	132.6	133.2	133.2	125.7	124.7
12	180.9	180.9	180.8	121.7	122.1
12a	132.4	133.6	133.7	136.5	137.1
13	27.5	24.9	22.8	30.0	30.4
14	171.3	170.9	170.5	170.6	170.0
15	52.5	52.3	52.6	–	52.2

(c) MS data for compounds VII, VB, IVC and VC. EIMS, electron impact mass spectroscopy.

Compound	EIMS (m/e) (relative intensity)
VII	398 (M^+ , 100), 380 (20), 362 (100), 331 (32), 321 (47)
VB	354 (M^+ , 100), 307 (60), 294 (88), 251 (8)
IVC	352 (M^+ , 100), 337 (90), 293 (70), 251 (20)
VC	366 (M^+ , 100), 351 (75), 293 (52), 251 (13)

pSY21d were identical, pSY15 was chosen as a vector for preparation of the last two constructs to allow the usage of suitable restriction sites for cloning.

Plasmid pSY15a, expressing *acmA* in addition to genes covering steps 1–5 (Fig. 3), caused the accumulation of auraviketone (VI) in TK24. Finally, the addition of the *SacI*–*AvrII* fragment carrying *dauE*, a C-7 ketoreductase from *S. peucetius*, to pSY15a generated pSY15b,

resulting in the production of auramycinone in *S. lividans* TK24. Auramycinone made up about 30% of the detectable metabolites.

DISCUSSION

According to the proposed biosynthetic pathway (Ylisonko *et al.*, 1996b), we speculated that a specific cyclase is needed to close each aromatic ring and 11

structural genes are required for an anthracyclonone pathway. The work presented here suggests, however, that nine structural genes are needed to form an anthracyclonone in *S. lividans*.

The host chosen for this study was *S. lividans* TK24 even though *Streptomyces coelicolor* CH999 has been frequently used in the investigation of aromatic polyketide metabolites (e.g. Zawada & Khosla, 1997; Fu *et al.*, 1996). CH999 has been genetically engineered to lack all the genes needed for actinorhodin biosynthesis (McDaniel *et al.*, 1993). Unfortunately, our attempts to produce metabolites with pSY15- or pSY21-based CH999 clones were not successful, since aromatic polyketides were not accumulated in the liquid cultures. Plasmids pSY21c- and pSY15-based constructs caused the formation of 20-carbon hybrid products in TK24 (Fig. 3, IVC, VC), whereas metabolites of 16 carbons (actinorhodin is built up from eight acetate units; Fig. 1) were not recovered. SnoA is an activator that presumably promotes expression of other polyketide genes in addition to nogalamycin biosynthetic genes. An expression plasmid comparable to pSY21c but containing the *ermE* promoter (Bibb *et al.*, 1985) in pIJ486 and not containing *snoA*, decreased the production of metabolites though hybrid products were not obtained in the culture extract. However, the yield was too low for structural analysis of the compounds (J. Kantola, unpublished results).

The production of stable compounds when oxygenase was present suggests that this enzyme is the last PKS component in forming the product, which is then released from the enzyme complex. The hybrid products obtained (Fig. 3, IVC, VC) sharing structural features of actinorhodin (Fig. 1) and nogalamycin were not oxygenated, suggesting that the reaction possibly directed at the *actVI* locus (Fernandez-Moreno *et al.*, 1994; Ichinose *et al.*, 1999) is competitive with that directed by the oxygenase. The factor that determines condensation of the second and third rings was not, however, clarified. The presence of oxygenase (or a corresponding enzyme) with the PKS may result in the correct orientation of a polyketide chain to allow condensation of two aromatic rings (B and C), suggesting that specific cyclases are not essential. This hypothesis, that no separate gene products are needed for the cyclizations, is consistent with the minimal construct being able to cause the production of aklanonic acid for daunomycin biosynthesis (Rajgarhia & Strohl, 1997; Gerlitz *et al.*, 1997). On the other hand, *dpsY*, recently identified in the daunomycin cluster, is probably essential for closing of the aromatic rings (Lomovskaya *et al.*, 1998), which is why we cannot rule out the possibility that cyclization activities were derived from TK24. Also DpsH has been mentioned to be involved in closing the second and the third rings (Gerlitz *et al.*, 1997), but recently published results by Lomovskaya *et al.* (1999), have suggested that DpsH is involved in daunosamine biosynthesis or its attachment to rhodomycinone. Since spontaneous cyclization reactions result in only minor amounts of products (generally less than 1%), the fact that auro-

mycinone was a major product (30% of the whole extract) suggests that an enzymatic reaction was involved.

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